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SIMILARITIES OF BIOPHYSICAL PROPERTIES OF SEVERAL HUMAN ENTEROVIRUSES AS SHOWN BY DENSITY GRADIENT ULTRACENTRIFUGATION OF MIXTURES OF THE VIRUSES.[1]

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SUMMARY

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Various mixtures of viruses representing the three types of poliovirus, ECHO 7 and Coxsackie A21, were ultracentrifuged in glycerol (or sucr:) density gradients and in CsCl density gradients. Visible bands from purified and concentrated viruses were fractionated and titers of each virus and ultraviolet adsorption measurements were determined. Appropriate antisera were employed to distinguish the viruses in the mixtures. Unconcentrated viruses did not produce visible bands, but distribution of viruses were determined after "blind sampling". In all cases tested the viruses showed very similar but not necessarily identical biophysical properties.

AUTHOR

INTRODUCTION

Brakke (1960) and de Duve et al. (1959) have reviewed the theory and techniques of denisty gradient ultracentrifugation. "Rate zonal" ultracentrifugation depends upon particle size and density, while equilibrium or isodensity ultracentrifugation depends upon the buoyant density of the particles, and the position of the band is independent of particle size. Interaction of virus particles with each other or the other components (water or the density enhancing component) of the solution may affect the results of either technique.

The results of numerous studies of physical as well as biological properties of human enteroviruses justify their classification as a group. The small differences in particle size or sedimentation characteristics of these viruses previously reported may be due to differences in techniques or conditions. The procedure to be described facilitates direct comparison of density and/or sedimentation velocity of 2 viruses without using carefully standardized conditions.

MATERIALS AND METHODS

Viruses. ECHO 7, strain Wallace; Coxsackie A-21, Strain Coe; and polioviruses, strains Mahoney, MEF1, and Saukett of types 1, 2, and 3, respectively, were propagated in HeLa Cells. Attenuated type 2 poliovirus, strain P712, was received from Dr. Albert Sabin and was propagated in primary monkey kidney and human amnion cultures. The viruses were purified by ultracentrifugation and banding in CsCl gradients as described elsewhere (Fromhagen and Martins, 1961; Mattern, 1962).

Antisera. Monkey antisera, specific for the appropriate viruses, were employed. The specificities were confirmed with respect to National Foundation reference antisera for the polioviruses and with the prototype virus for ECHO 7 (Wallace strain).

Virus assay. Poliovirus was assayed by the plaque technique as described elsewhere (Schaffer, 1962) and expressed as plaque forming units (pfu). ECHO 7 was assayed in the same manner as poliovirus except that 4 days rather than 3 were allowed for plaque development. Coxsackie A21 (Coe) was assayed by tube titration (Fromhagen and Martins, 1961)[2]. Antiserum, when employed, was incubated with diluted virus at room temperature for 1 hour prior to inoculation. The antiserum was used at a dilution that suppressed at least 99.9% of the infectivity.

Isodensity centrifugation. Virus samples, including dilute buffer, were mixed with a concentrated solution of CsCl to give a final density of about 1.34. They were then centrifuged at 30,000 rev/min in a Spinco SW-39 rotor for 18 to 24 hours to establish the gradient and form a virus band.

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2. Numerous attempts were made to assay Coxsackie A21 by the plaque technique, but the results were not reproducible.

Density gradient sedimentation. Virus samples were layered above previously prepared density gradients and centrifuged 2 to 3 hours in a Spinco SW-39 rotor at 30,000 rev/min to obtain virus bands. Glycerol as well as sucrose, was employed as a density enhancing medium. Concentrations of glycerol of 81,66,51,37, and 21% by weight were used to form gradients similar in density and viscosity with the previously described sucrose gradients (Schwerdt and Schaffer, 1956). NaCl was omitted, but the sucrose or glycerol solutions were buffered with 0.01-0.02 M phosphate, pyrophosphate, or tris, pH 7.1-7.5.

Sampling from density gradients. A simple device, shown in Fig. 1, was constructed to facilitate sampling of CsCl and glycerol (or sucrose) gradients[3]. Mineral oil was added to each density gradient tube (usually prior to centrifugation) to minimize entrapment of air. The tube was attached to the device while the stopcock was open to the reservoir, forcing out most of the remaining air. Bands of light scattering material were visualized with the aid of spotlamp illumination from above. A hole was punctured in the bottom of the tube with a needle and the stopcock was turned to connect the tube with the displacement syringe which previously had been filled with oil from the reservoir. Samples were collected from the hole in the bottom of the tube as measured volumes of oil were introduced at the top from the displacement syringe. The viscocity of the oil prevented rapid flow. This device facilitated the sampling of volumes ranging from less than one drop to greater than 1 ml. Selection of sample size was predicated upon appearance of bands and purpose of the experiment.

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3. A similar device may now be obtained from Microchemical Specialties Company, Berkeley, California.

Ultraviolet absorption. Individual samples from the density gradients were measured vs. appropriate blanks at 260 mμ (Schwerdt and Schaffer, 1956) and the ultraviolet absorption was expressed as OD_{260}.

RESULTS

Ultracentrifugation of mixtures of viruses in sucrose or glycerol density gradients. Each mixture of two human enteroviruses tested produced a single band when sedimented into a glycerol or sucrose gradient. The total amount of recovered virus corresponded well with the input virus. No more than a few per cent of the viruses recovered were outside the region of the bands. Separation (either partial or complete) of viruses would result in a change in relative concentration of the virus at certain positions within the gradient. When the ratios of infectivities of the two viruses were compared for various regions, i.e., outside the band, the peak, and the shoulders of the band, no significant differences were observed.

Figure 2 shows the results of sedimentation of a mixture of MEF1 poliovirus and ECHO 7 virus, with titrations in the presence of the appropriate antisera. The results obtained upon centrifugation of a mixture of approximately equal quantities (based upon ultraviolet absorption) of Mahoney and Coe viruses is shown in Fig. 3. Although a single sharp band was observed visually, the distribution of virus among the fractions did not show a sharp peak, indicating possible disturbance during sampling. The infectivity results represent preliminary

plaque titrations performed without antiserum. Figure 4 shows only the ultraviolet absorption results corresponding to the single band of another mixture of Mahoney and Coe viruses. Reliable plaque assays were not obtained for these fractions[2], but tube titrations were made on selected fractions. Results are shown in Table 1. Although these assays are not as precise as plaque assays, the results indicate that infectivities of both viruses ~~are~~ were associated with the observed band.

Fig. 5 demonstrates the identity of sedimentation characteristics of proflavine-containing and normal polioviruses (Schaffer, 1962). A small quantity of normal virus was mixed with virus propagated in the presence of proflavine which had low residual infectivity due to exposure to light. The distribution, both of fluorescence (as measured in an Aminco-Bowman spectrophotofluorometer) due to the dye-containing particles and of infectivity due to dye-free particles corresponded with the distribution of ultraviolet absorption.

Isodensity ultracentrifugation of mixtures of viruses in CsCl density gradients. Buoyant densities of the enteroviruses were found to be very similar. In most, but not all cases, single bands were observed by visualization of light scattering and distributions of infectivity and ultraviolet absorption among fractions. Figure 6 shows the results of measurements following centrifugation of a mixture of Saukett and MEF1 polioviruses. Fig. 7 shows the distribution of ultraviolet absorption obtained from a single visible band following ultracentrifugation of a mixture of Coe and Mahoney viruses. Plaque assay results were not available for this experiment.

A partial separation of MEF1 and ECHO 7 viruses occurred when the mixture was centrifuged in a CsCl solution (Fig. 8). Visually, the band appeared double in contrast to the single sharp bands in the previous experiments. Ultraviolet absorption correlated with total infectivity, but neither showed a double peak. The lack of a double peak was, no doubt, due to lack of resolution in taking samples as large as 0.2 ml each. A more sensitive indication of the presence of the double peak is seen in the ratio of infectivities which showed over a hundredfold difference between fraction 1 and fractions 3 and 4. The intervening fraction 2, as well as the small amount of infectivity remaining in the upper and lower portions of the tube, showed no significant differences in ratios from the ratio for the original mixture. Had equilibrium been reached, the difference in densities, $\Delta\rho$, between two fractions of 0.2 ml each could be calculated to be approximately 0.009 gm/cm^3 (Trautman, 1960; Ifft et al., 1961). Actually, insufficient time (21 hrs in this experiment) was allowed for equilibrium to be reached. Pycnometric measurements in an independent experiment indicated the actual value for $\Delta\rho$ was 0.005 at 21 hrs. The possible significance of small differences in buoyant density will be discussed below.

In addition to application of CsCl gradient centrifugation to mixtures of purified viruses, the technique was applied to unconcentrated viruses in tissue culture fluids. These fluids were clarified by low speed centrifugation to remove cell debris prior to use. Figure 9 shows the results of such an experiment with MEF$_1$ and Mahoney viruses. Figure 10 shows a similar experiment with Saukett and the attenuated P712 strain.

No band was visible in either experiment, but "blind sampling" from the center portion of each tube revealed that each pair did indeed give a single band[4].

DISCUSSION

Both the isodensity and moving band density gradient ultracentrifugation procedures have proven useful as preparative procedures for viruses in our laboratories as well as those of others. These procedures physically characterize the entity responsible for infectivity. In the present study we have employed these techniques as a direct method to demonstrate similarities of members of the human enterovirus group. Results of independent investigations employing electron microscopy, sedimentation velocity measurements and buoyancy in CsCl have, in general, indicated that the members of this group are similar in size and density and thus presumably in nucleic acid content. Independent measurements depend upon standarization of conditions. Our experiments provide a direct confirmation of the similarities of these properties for those viruses tested. The techniques also provide a means for detecting relatively small differences in physical properties between viruses.

At any given time, the position of a band of virus moving down through a gradient of density less than that of the virus is related to the sedimentation velocity of the virus (Brakke, 1960).

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[4]. Visibility of a band is a function of concentration of physical particles. The specific infectivities (pfu/OD_{260} ratios) of most of the purified preparations used in prior experiments were low, whereas some of the tissue culture fluids employed here were of high titer, accounting for the difference in visibility despite comparable infectivity titers.

We conclude that both Coe and ECHO 7 viruses have sedimentation velocities of about 160 S, corresponding with the value obtained for poliovirus by analytical ultracentrifugation (Schaffer and Schwerdt, 1959), since each of these viruses sedimented along with poliovirus in a sucrose or glycerol gradient.

Hanzon and Philipson (1960) reported the diameter of ECHO 7 virus to be about 20 mμ based upon electron microscopy of partially purified preparations. They also stated that ECHO 7 had a sedimentation coefficient of 40S, but the details of this work are as yet unpublished. In contrast to such unusually small values, our results show this virus to have sedimentation characteristics comparable to the other enteroviruses. Preliminary electron micrographs of our preparations of ECHO 7 virus showed a particle size similar to that of Coe virus observed by Frommhagen and Martins (1961).

The position of a band of virus in a CsCl gradient, after prolonged centrifugation, corresponds to the buoyant density of the virus. This is dependent not only upon the dry density, which in turn is related to composition, but also upon hydration of the particles, interaction with or permeability to CsCl, and even factors such as compressibility of the solvent and the particles (Hearst et al, 1961). The distribution of a homogeneous component within a band at equilibrium is dependent upon diffusion coefficient. No conclusions may be drawn from virus distributions in our experiments because the duration of centrifugation allowed for establishment of a gradient and banding of the virus, but not for establishment of equilibrium, and also because of possible minor disturbances during decelleration and sampling.

A small difference in the positions of bands of two viruses, as seen in Fig. 8, is most likely due to factors such as hydration mentioned above. Similarly, small or partial separations have been observed with a few other preparations, including single batches of purified MEF1 poliovirus. In the latter instance, a biological difference, time of plaque appearance was related to the physical difference (Schaffer, unpublished). Attardi and Smith (1962) observed a partial separation of poliovirus infectivity from ultraviolet absorption in RbCl density gradient centrifugation. The purpose of the present report is to point out similarities among the enteroviruses rather than to elucidate minor differences. Further work will be necessary to establish the significane of such differences. Preliminary experiments on CsCl gradient centrifugation of another small RNA virus, vesicular exanthema of swine virus, showed a band at 1.37 gm/cm^3. Some experiments indicated a partial separation of infectivity from the majority of the physical particles (F.L. Schaffer and M.E. McClain, unpublished), while other experiments showed two distinct bands of infectious virus (Shoemaker et al, 1963, and unpublished results). Roizman and Roane (1961) observed a small but significant difference in density between 2 strains of herpes simplex, a DNA virus, upon centrifugation of mixtures of strains in CsCl. This difference may be due to the surface properties of these strains (Roizman and Roane, 1963).

Ultracentrifugation of mixtures of viruses followed by fractionation and titration in the presence of antisera or in selective hosts appears to be particularly useful in physical characterization of viruses. In addition to the "blind sampling" experiments presented in Figs. 9 and 10, a purified

virus was used in other experiments (not shown) as a marker in a mixture with tissue culture fluid virus. After harvest of the marker band, titrations were performed with and without antiserum to the marker virus. Antiserum to the "unknown" (tissue culture fluid) virus was unnecessary since its contribution to the total infectivity was insignificant. Other variations, such as a radioactive tracer labeled virus as a marker, could also be employed.

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TABLE 1

INFECTIVITY OF SELECTED FRACTIONS FROM GLYCEROL GRADIENT CENTRIFUGATION OF A MIXTURE OF COE AND MEF1 VIRUSES. TUBE TITRATION WITH HELA CELLS

Fraction	Antiserum	Log $TCID_{50}$/ml
3	- -	7.0
	Coe	6.5
	Polio	7.0
	Coe + Polio	<6.0
5	- -	8.0
	Coe	6.8
	Polio	7.3
	Coe + Polio	<6.0
8	- -	7.2
	Coe	6.5
	Polio	6.0
	Coe + Polio	<6.0

FIGURE LEGENDS

Fig. 1. Device for removing samples from density gradient tubes.

A. Density gradient tube.

B. Transparent plastic block machined to fit density gradient tubes and stopcock.

C. Metal stopcock with Luer-Lok syringe connections.

D. Displacement syringe (1, 2, or 5 ml).

C. Mineral oil reservoir (Large syringe without plunger).

F. Light beam.

Fig. 2. Glycerol density gradient sedimentation of a mixture of MEF1 and ECHO 7 viruses. Mixture of 6.3×10^8 pfu of MEF1 and 2.2×10^8 pfu of ECHO 7 in 0.57 ml with $OD_{260} = 0.412$ layered over glycerol gradient. Five fractions of 0.1 ml each were taken in the region of the observed band. Plaque assays were in the presence of appropriate antisera.

(In this and subsequent figures, numbering of fractions was from the lower toward the upper portion of the tube, and percent total = $\frac{\text{amount in individual fraction} \times 100}{\text{sum in all fractions}}$.)

Fig. 3. Sucrose density gradient sedimentation of a mixture of Mahoney and Coe viruses. Total infectivity of mixture approximately 10^9 pfu in 0.6 ml and $OD_{260} = 1.10$ layered over sucrose gradient. Ten fractions of 0.04 ml each were taken in the region of the observed band and diluted six-fold.

Fig. 4. Glycerol density gradient centrifugation of mixture of Mahoney and Coe viruses. Volume of mixture = 0.43 ml and OD_{260} approx. 2.0. Twelve fractions of 0.04 ml each were taken from the region of the observed band and diluted six-fold.

Fig. 5. Glycerol density gradient sedimentation of a mixture of photoinactivated proflavine-containing MEF1 and ordinary MEF1 viruses. Two tenths ml of mixture was layered over a glycerol gradient in a 2 ml centrifuge tube. Infectivity of mixture = 4×10^8 pfu, OD_{260} = 1.9, of which 70% was due to proflavine virus. Single drop fractions were collected by gravity flow from the region of the observed fluorescent band; each drop being diluted into 0.2 ml of buffer for assay.

Fig. 6. CsCl isodensity centrifugation of a mixture of MEF1 and Saukett viruses. Infectivity = 1.5×10^{10} pfu of each virus in a total of 1.6 ml of CsCl solution, the remainder of the tube being filled with mineral oil. OD_{260} of mixture = 0.41. Fourteen fractions of 0.04 ml each were collected from the region of the observed band and diluted six-fold for assay. Infectivity was assayed on fractions 4 through 10 only employing appropriate antisera. Solid line = infectivity, dashed line = ultraviolet absorption.

Fig. 7. CsCl isodensity centrifugation of a mixture of Coe and Mahoney viruses. Mixture which was added to CsCl solution, and sampling were as described for Fig. 4.

Fig. 8. CsCl isodensity centrifugation of a mixture of MEF1 and ECHO 7 viruses, showing a slight separation of the two viruses. Infectivity of mixture approximately 8×10^8 pfu of MEF1 and 3×10^8 pfu of

ECHO 7, OD_{260} = 0.110 of which approximately 65% was contributed by MEF1. Five fractions of 0.2 ml each were taken from the region of the band(s). Plaque assays were performed in the presence of appropriate antisera.

Fig. 9. CsCl isodensity centrifugation of a mixture of unpurified (tissue culture fluid) MEF1 and Mahoney viruses. Total infectivity approximately 4×10^7 pfu of MEF1 and 5×10^8 pfu of Mahoney. Sixteen fractions of 0.05 ml each were collected from the center portion of the tube. Plaque assays were in the presence of appropriate antisera.

Fig. 10 CsCl isodensity centrifugation of a mixture of unpurified P712 and Saukett polioviruses. Total infectivity approximately 9×10^6 pfu of P712 (assayed on primary human amnion cultures) and 3×10^8 pfu of Saukett. Fractions taken as described for Fig. 9.

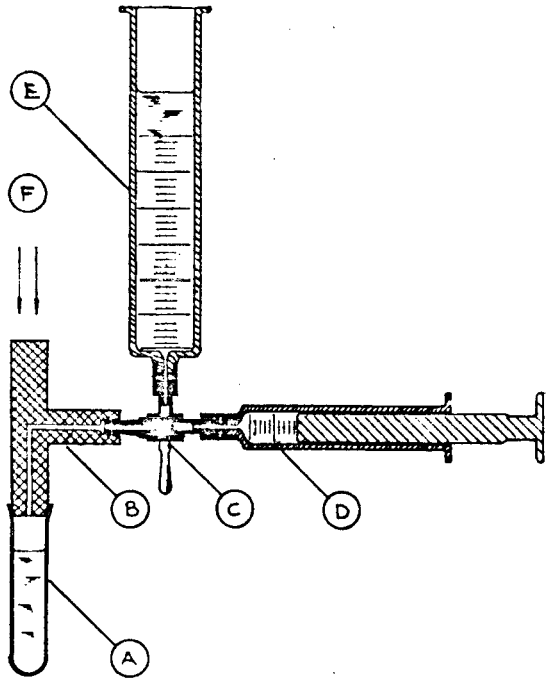

FIG. 1. Device for removing samples from density gradient tubes.
A. Density gradient tube.
B. Transparent plastic block machined to fit density gradient tubes and stopcock.
C. Metal stopcock with Luer-Lok syringe connections.
D. Displacement syringe (1, 2, or 5 ml).
E. Mineral oil reservoir (large syringe without plunger).
F. Light beam.

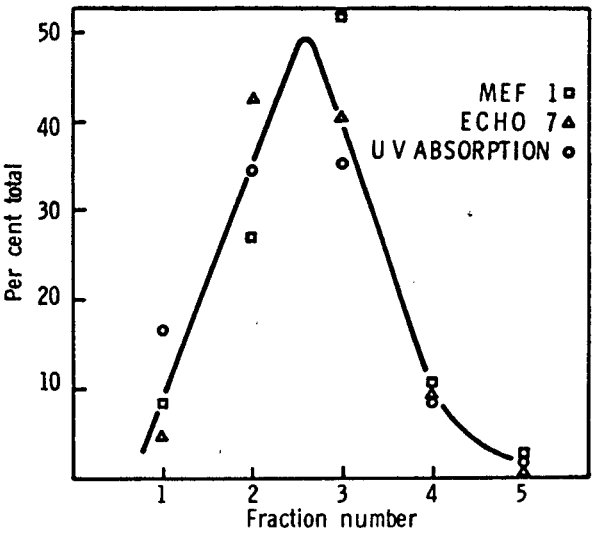

FIG. 2. Glycerol density gradient sedimentation of a mixture of MEF 1 and ECHO 7 viruses. Mixture of 6.3×10^8 pfu of MEF 1 and 2.2×10^8 pfu of ECHO 7 in 0.57 ml with OD_{260} = 0.412 layered over glycerol gradient. Five fractions of 0.1 ml each were taken in the region of the observed band. Plaque assays were in the presence of appropriate antisera. (In this and subsequent figures, numbering of fractions was from the lower toward the upper portion of the tube.)

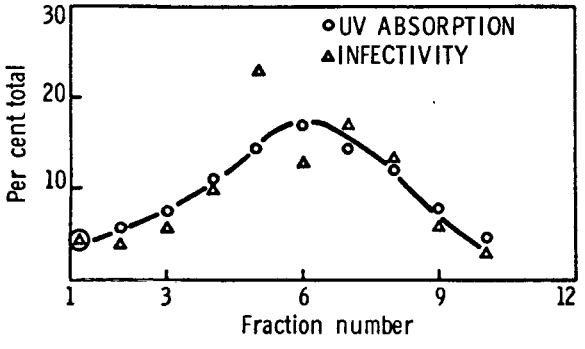

FIG. 3. Sucrose density gradient sedimentation of a mixture of Mahoney and Coe viruses. Total infectivity of mixture approximately 10^9 pfu in 0.6 ml and OD_{260} = 1.10 layered over sucrose gradient. Ten fractions of 0.04 ml each were taken in the region of the observed band and diluted 6-fold.

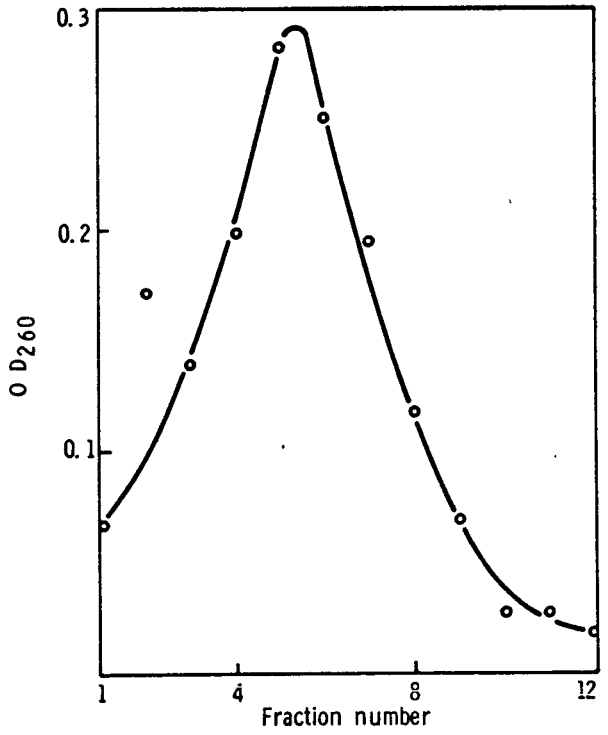

FIG. 4. Glycerol density gradient centrifugation of mixture of Mahoney and Coe viruses. Volume of mixture - 0.43 ml and OD_{260} approx. 2.0. Twelve fractions of 0.04 ml each were taken from the region of the observed band and diluted 6-fold.

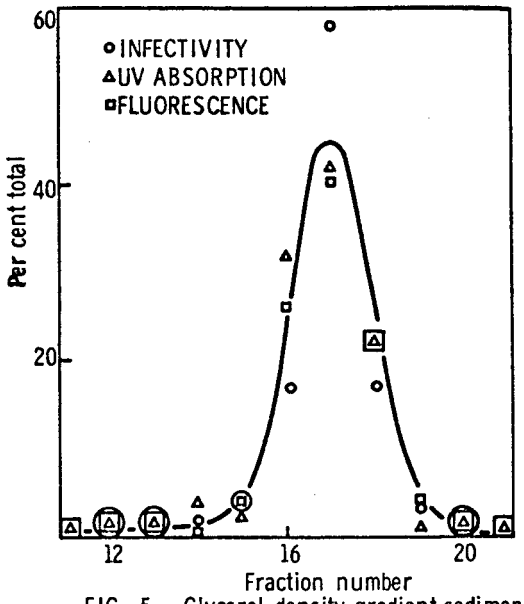

FIG. 5. Glycerol density gradient sedimentation of a mixture of photoinactivated proflavine-containing MEF 1 and ordinary MEF 1 viruses. Two-tenths ml of mixture was layered over a glycerol gradient in a 2 ml centrifuge tube. Infectivity of mixture 4 x 10^8 pfu, OD_{260} = 1.9, of which 70% was due to proflavine virus. Single drop fractions were collected by gravity flow from the region of the observed fluorescent band, each drop being diluted into 0.2 ml of buffer for assay.

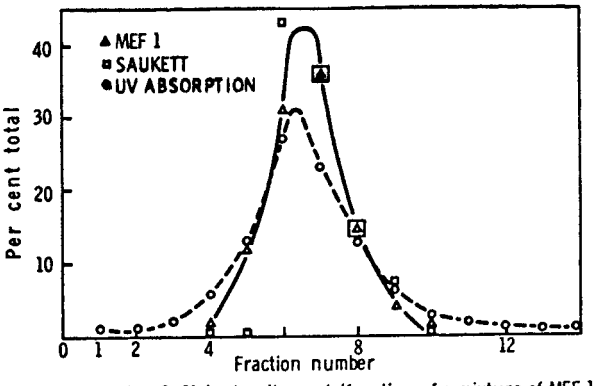

FIG. 6. CsCl isodensity centrifugation of a mixture of MEF 1 and Saukett viruses. Infectivity = 1.5 x 10^{10} pfu of each virus in a total of 1.6 ml of CsCl solution, the remainder of the tube being filled with mineral oil. OD_{260} of mixture = 0.41. Fourteen fractions of 0.04 ml each were collected from the region of the observed band and diluted 6-fold for assay. Infectivity was assayed on fractions 4 through 10 only employing appropriate antisera. Solid line = infectivity, dashed line = ultraviolet absorption.

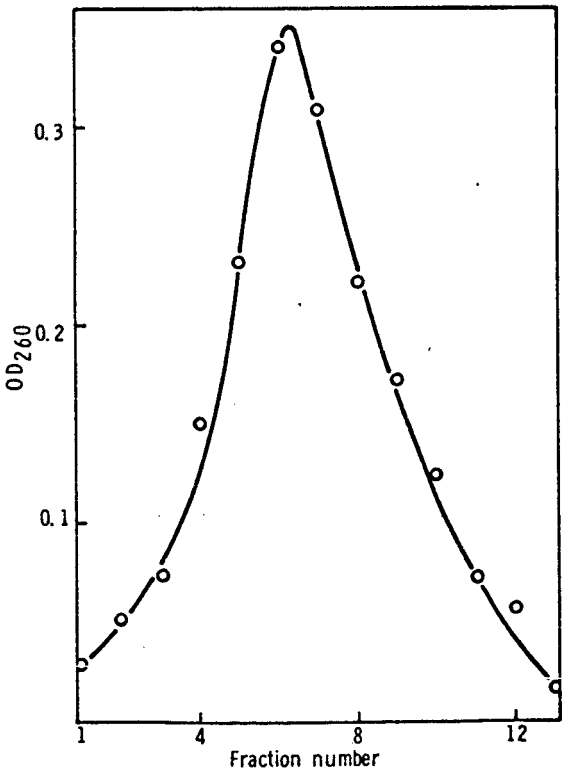

FIG. 7. CsCl isodensity centrifugation of a mixture of Coe and Mahoney viruses. Mixture which was added to CsCl solution, and sampling were as described for Fig. 4.

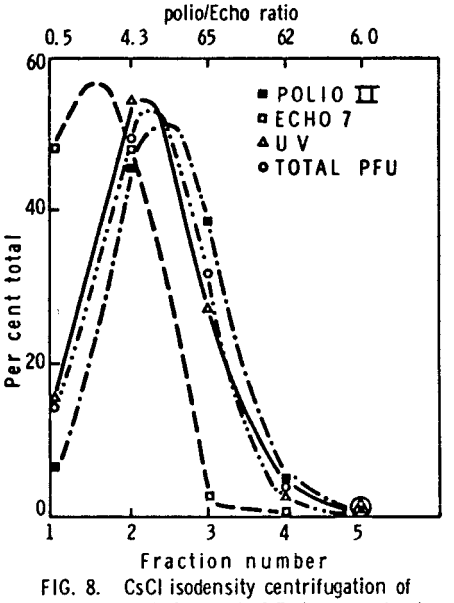

FIG. 8. CsCl isodensity centrifugation of a mixture of MEF 1 and ECHO 7 viruses, showing a slight separation of the two viruses. Infectivity of mixture was 1.6 x 10^9 pfu of MEF 1 and 4.8 x 10^8 pfu of ECHO 7, OD_{260} = 0.118 of which approximately 65% was contributed by MEF 1. Five fractions of 0.2 ml each were taken from the region of the band(s). Plaque assays were performed in the presence of appropriate antisera. Ratios of infectivity of fractions are shown; not shown are MEF 1/ECHO 7 ratios of 3.3, 4.7, and 4.8 for original mixture, bottom, and top portions of tube respectively.

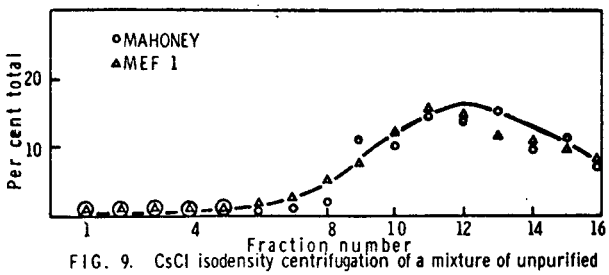

FIG. 9. CsCl isodensity centrifugation of a mixture of unpurified (tissue culture fluid) MEF 1 and Mahoney viruses. Total infectivity approximately 4×10^7 pfu of MEF 1 and 5×10^8 pfu of Mahoney. No band was visible, but 16 fractions of 0.05 ml each were collected from the center portion of the tube. Plaque assays were in the presence of appropriate antisera.

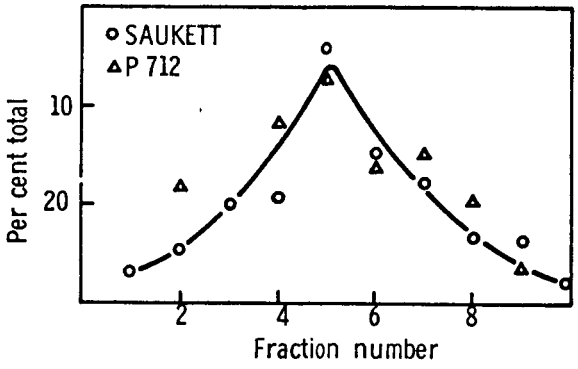

FIG. 10. CsCl isodensity centrifugation of a mixture of unpurified P 712 and Saukett polioviruses. Total infectivity approximately 9×10^6 pfu of P 712 (assayed on primary human amnion cultures) and 3×10^8 pfu of Saukett. Samples taken as described for Fig. 9.